The first wave of B lymphopoiesis develops independent of stem cells in the murine embryo

Short title: HSC-independent B-1 cell lineage

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Abstract

In the developing mouse embryo, there are several waves of hematopoiesis. Primitive and definitive erythro-myeloid lineages appear prior to hematopoietic stem cell (HSC) emergence and these waves are considered to be transient, supporting embryonic homeostasis until HSC-derived hematopoiesis is established. However, recent evidence strongly supports that HSC-independent immune cells such as tissue macrophages and some innate lymphoid cells develop in the mouse embryo and persist into post natal life. Innate type B-1 cells have also been reported to emerge from hemogenic endothelial cells in the extra-embryonic yolk sac (YS) and para-aortic splanchnoleura (P-Sp), and continue to develop in the fetal liver even in HSC-deficient mouse embryos. This review examines B-1 cell development in the context of the layered immune system hypothesis of B lymphopoiesis and emergence of B-1 cells independent of HSC.

Hemogenic endothelial cell-derived hematopoiesis in the developing mouse embryo

The first blood cells in the mouse embryo are observed in the embryonic day (E) 7.5 yolk sac (YS)[1, 2]. These blood cells are mainly large erythroblasts, expressing embryonic type hemoglobin molecules, and are called primitive erythrocytes. Primitive types of megakaryocyte and macrophage progenitors are also produced at this stage (primitive hematopoiesis). Around E8.0, erythroblasts that are much smaller than the primitive erythrocytes and express adult type hemoglobin molecules (definitive erythroid progenitors) are detectable together with myeloid cells arising from erythro-myeloid progenitor cells (EMPs). These EMPs are produced mainly in the YS[3]. Finally around E11, the first adult-repopulating HSCs are detectable within the aorta-gonado-mesonephros (AGM) region[4, 5].

The first HSCs are considered to "bud" from endothelial cells (ECs) residing in the ventral side of the dorsal aorta that are called "hemogenic ECs"[6-9]. Hemogenic ECs are blood-producing ECs and the evidence of their actual presence and function is provided by in vitro and in vivo assays[10-15]. For example, in vivo lineage tracing studies using vascular endothelial cadherin promoter driven Cre recombinase (VEcad-cre) bred to Rosa-yellow fluorescent protein (YFP) mice, all the hematopoietic cells in the adult bone marrow (BM) are YFP positive, indicating that all the blood cells were endothelial derived[12, 15]. *Runx1* is a critical transcriptional factor for hematopoiesis. *Runx1* knockout embryos die at E12.5 lacking detectable EMPs, blood cell budding in the dorsal aorta, and transplantable HSCs. When *runx1* is deleted under the control of an endothelial specific VE-cadherin promoter, knockout embryos display similar phenotypes to total runx1 knockout embryos, such as loss of EMPs and HSCs, while *runx1* deletion under a hematopoietic specific vav promoter does not influence EMP and HSC production. These results indicate that EMP and HSCs are derived from hemogenic ECs and that *runx1* is indispensable for the endothelial-hematopoietic transition that occurs in E8 to E11.5 murine embryos[12, 16]. Of note, although primitive erythroblasts express *runx1*, primitive hematopoiesis is not affected by

runx1 deletion[17]. Whether primitive hematopoiesis is derived directly from mesoderm or from hemogenic EC is still a controversial topic[2].

Once HSCs emerge in the AGM region, these HSCs seed the fetal liver where they dramatically expand, and then migrate to spleen and BM just before birth. HSCs settle in the BM to maintain hematopoiesis throughout life, self-renewing and producing all blood cell lineages.

A layered Immune system hypothesis and B-1 cell development

As mentioned above, there are several waves of hematopoietic cell emergence during embryogenesis including primitive hematopoietic progenitors, EMPs, and HSCs. The first two waves (primitive and EMPs) are considered to be transient and not to contribute to postnatal hematopoiesis, however, recent studies have revealed that tissue macrophages are of fetal origin and are not replenished by adult HSC-derived monocytes during homeostasis[18]. Similarly, some lymphoid populations are considered to be of fetal origin, challenging the stem cell theory that all the lymphoid cells are derived from HSCs.

The layered immune system hypothesis was proposed by Drs. Herzenberg and Herzenberg in 1989[19], attempting to explain why certain types of lymphoid cells were preferentially produced from fetal stem/progenitors, but not from adult stem/progenitor cells. In this model, there are at least 2 waves of B cell development during embryonic to neonatal periods; the first one is the development of CD5+ B cells, called B-1a cells, produced predominantly by fetal liver stem/progenitor cells. Following the first wave, the second wave of conventional B (B-2) cell development produced by HSCs starts at fetal liver stage. The second wave becomes predominant in the postnatal BM and lymphoid organs whereas the first wave wanes with advancing age. Thus, these 2 waves are over lapping, detectable at fetal liver stage (B-1>B-2), but seem to originate from different stem/progenitor cells arising at different times during development. B-1 cells are innate immune B cells that can be distinguished from conventional B-2 cells in the peripheral lymphoid organs. B-1 cells mainly reside in the pleural and peritoneal

cavities and produce natural IgM antibodies against stereotypic antigens independent of T cell help. In addition to their specific surface markers ($IgM^{hi}IgD^{lo}CD11b^{+}CD5^{+/-}$), one of the unique characteristics of B-1 cells is their different developmental pathway from conventional B-2 cells[20]. It has been recognized that B-1 cells are readily reconstituted by fetal liver progenitor cells upon transplantation, whereas adult BM cells do not repopulate this lineage. The "pro-B cell" ($B220^{+}CD43^{+}$) population isolated from E16 fetal liver reconstitutes $CD5^{+}$ B-1a cells efficiently, but fails to produce B-2 cells in recipient mice upon transplantation, while the pro-B cell population from adult BM shows the opposite results[21]. In IL-7 knockout mice, B lymphopoiesis is observed exclusively in fetal and neonatal periods, but ceases after 7 weeks of age[22]. The B cells found in IL-7 knockout mice belong to the peritoneal B-1 and splenic marginal zone B cell pools and persist throughout adult life without being replenished from BM derived B progenitor cells (as these progenitors are no longer present). Similarly, when *Rag2* gene expression is ablated at birth in conditional knockout mouse, $CD5^{+}$ B-1a cells in the peritoneal cavity and marginal zone B cells in the spleen are maintained although splenic Follicular (FO) B cells (known to represent B-2 cells) are not produced[23]. These findings have supported the hypotheses that progenitors that give rise to $CD5^{+}$ B-1a cells are present only in fetal and neonatal life, and that $CD5^{+}$ B-1a cells display self-renewal properties. Identification of $AA4.1^{+}CD19^{+}B220^{lo\text{-}neg}$ B-1 specific progenitor cells in the fetal liver and neonatal BM support this hypothesis[24]. A recent report that a purified long-term adult HSC is unable to reconstitute B-1a cells upon single cell HSC transplantation has also given strong evidence for these hypotheses[25]. Interestingly, since the first phenotypic B-1 progenitor cells are detectable at E10.5 in the AGM region, at a time when the first HSCs emerge[26], this raises an intriguing question as to the origin of the B-1 progenitor cells; it would be impossible for a HSC (that has been just born) to self-renew/expand and differentiate into B-1 progenitor cells simultaneously. We and others have hypothesized that precursor cells other than HSCs must have produced the B-1 progenitor cells prior to HSC emergence in order for both cell types to appear concomitantly.

Hemogenic endothelial origin of B-1 cells

Evidence for B lymphoid potential detectable in the YS, para-aortic spranchnopleura (P-Sp, later called as AGM after E10) has been controversial: Cumano et al found B and T lymphoid potential only in the P-Sp and not YS prior to HSC emergence[27]. Yokota et al demonstrated Rag1^{+} lymphoid cell production from P-Sp Tie2^{+} endothelial cells (ECs), but not from YS CD41^{+} cells[28]. Nishikawa et al showed B and T lymphoid potential in CD45^{-}VEcad^{+} ECs from both E9.5 YS and P-Sp[11]. Since circulation is already established as early as E8.25, such discrepant results have been attributed to the fact that the circulation may have mixed lymphoid progenitor cells derived from different hematopoietic tissues and obscured the site of origin of these cells. In order to clarify the temporal and spatial origin of the first B cells produced, we have utilized a *Ncx1* knockout mouse model, where the embryonic heart develops normally but never begins beating[29]. While the lack of a heartbeat is embryonic lethal at E11, the embryos survive until E10.5 without any systemic blood cell circulation[3]. When E9 YS and P-Sp cells from wild type and Ncx1$^{-/-}$ embryos were plated on OP9 stromal cells that support B lymphopoiesis. AA4.1^{+}CD19^{+}B220^{dim} B-1 progenitor cells were detected at 8 days of coculture, and by 14 days of culture, CD19^{+}B220^{+} B cells were expanded from both wild type and Ncx1$^{-/-}$ YS and P-Sp. These CD19^{+}B220^{+} cells matured into IgM^{+} B cells and secrete anti-phosphorylcholine antibodies (that are thought to be preferentially secreted by B-1 cells) in vitro. When YS/P-Sp derived CD19^{+}B220^{+} cells were transferred into the peritoneal cavity of NOD/SCID/IL2γc$^{-/-}$ 2 day old neonates, these B cells matured into only peritoneal B-1 cells and splenic MZ B cells, but not B-2 cells. These AA4.1^{+}CD19^{+}B220^{+} cells were also produced from ECs as early as the 4-somite pair stage of YS and P-Sp development (before the heart normally begins beating). Importantly, these B cells were produced from VEcad^{+}CD41^{-} hemogenic ECs, but not from CD41^{+}c-kit^{+} hematopoietic cells until the 26-somite pair stage, suggesting that the lymphoid progenitor cells were derived via an endothelial to hematopoietic transition. These findings demonstrated that the first wave of B cells belongs to the

B-1 cell lineage and are produced from hemogenic ECs in YS and P-Sp, prior to HSC emergence[29].

The layered immune system hypothesis has recently been updated by Drs. Montecino-Rodriguez and Dorshkind[30]. They proposed that the first wave of B lymphopoiesis is comprised of YS/P-Sp hemogenic EC-derived B-1 cells, followed by a second wave of fetal liver HSC/progenitor-derived B cell production. In the second wave, B-1 lineage cells are mainly produced and B-2 lineage cell production initiates. In the third wave, BM HSCs produce predominantly B-2 lineages cells and the B-1 lineage cell production wanes along with aging. At the time this model was proposed, evidence for the first wave had been demonstrated only by *in vitro* culture and cultured cell transplantation, showing the "potential" of YS/P-Sp-derived cells to become B-1 precursors. In order to examine whether the first B-1 lineage production prior to HSC emergence is actually happening *in vivo*, we have utilized unique transgenic embryos that lack HSCs, but harbor EMP and a few B and T cells in the fetal liver[31, 32].

B-1 cells develop in the HSC deficient embryos.

Core-binding factor beta (CBF*β*) is the common non-DNA-binding subunit of the Cbf family of heterodimeric transcription factors and associates with CBFα subunits to increase the affinity of CBFα DNA-binding. CBF*β* is required for CBFα2 (also called runx1) function and loss of CBF*β* leads to embryonic lethality at E11-13, similar to *runx1*$^{-/-}$ embryos, displaying a severe deficiency of EMP and complete loss of HSCs[31, 33]. However, EMP formation and rare B and T cells were recovered in the fetal liver of *Cbfβ*$^{-/-}$ embryos when *Cbfβ* was expressed in *Cbfβ*$^{-/-}$ embryos under control of the endothelial Tek regulatory sequence as a transgene[31, 32]. Since *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos survive for at least one day after birth and yet completely lack HSCs, we anticipated this complex transgenic murine system would be a great tool to analyze the phenotype and function of the B cell development that occurs in the absence of HSCs. We hypothesized that

the previously unclassified B cells found in the HSC-deficient *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* fetal liver were cells of the B-1 lineage[34]. As expected, AA4.1^{+}CD19^{+}B220$^{lo\text{-}neg}$ B-1 specific progenitor cells were detected In the fetal liver and spleen of *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos. In wild type embryos, AA4.1^{+}CD19^{+}B220$^{lo\text{-}neg}$ B-1 progenitor cells become CD19^{+}B220^{+}CD43^{+} pro-B cells in the fetal liver, which subsequently differentiate into B-1 cells in the recipient peritoneal cavity upon transplantation[21]. However, in *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos, B220 was not up-regulated in AA4.1^{+}CD19^{+} population and CD19^{+}B220^{+}CD43^{+} pro-B cells was not detected in the fetal liver throughout embryonic life, suggesting delayed maturation of B-1 progenitors in mutant embryos. When the fetal liver AA4.1^{+}CD19^{+}B220$^{lo\text{-}neg}$ cells from E17.5 wild type and mutant embryos were co-cultured with OP9 stromal cells with IL-7 and Flt3-ligand, wild type AA4.1^{+}CD19^{+}B220$^{lo\text{-}neg}$ cells expanded in 10 days up to 1M cells and matured into CD19^{+}B220^{+} (and some IgM^{+}) pro B cells. On the other hand, mutant AA4.1^{+}CD19^{+}B220$^{lo\text{-}neg}$ cells did not expand as much as wild type cells and it took 17 days to mature into CD19^{+}B220^{+} pro B cells phenotype. In addition, most of the cells become Mac1^{+} myeloid cells instead of B cells. Since AA4.1^{+}CD19^{+}B220$^{lo\text{-}neg}$ B-1 progenitor cells are originally reported as myeloid-B-lymphoid biopotent progenitors[35], the B cell potential in this population seems to be altered in mutant embryos.

When fetal liver mononuclear cells from wild type and *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos were transplanted into NOD/SCID/IL2γc$^{-/-}$ neonates, wild type fetal liver cells reconstituted all the myeloid, T, B cell lineages, including peritoneal B-1a, B-1b, B-2, and spleen FO, MZ, and B-1 cells. On the other hand, *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* fetal liver cells repopulated only peritoneal B-1a, B-1b and splenic MZ B cell at very low frequencies[34]. Since no B-2 and T cells were reconstituted, it was clear that no HSCs exist in *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos.

These data indicate that functional B-1 progenitor cells are present in the HSC-deficient fetal liver, but their expansion and maturation abilities seems to be impaired in *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos. We hypothesized that this impairment may be due to the lack of *Cbfβ*

expression in B-1 progenitor cells. In *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos, *Cbfβ* expression is rescued only in TEK expressing ECs; once blood cells are produced from the TEK^{+} hemogenic EC, blood cells lose TEK and *Cbfβ* expression. In order to test this hypothesis, *Cbfβ* was overexpressed by retrovirus vector infection of *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* fetal liver mononuclear cells and these cells were transplanted into NOD/SCID/IL2γc$^{-/-}$ neonates[34]. *Cbfβ* expression significantly improved B-1 and MZ B cell engraftment of mutant fetal liver mononuclear cells up to 50% chimerism in the peritoneal cavity and spleen of recipient pups, but never rescued other lineages, such as B-2 or T cells, demonstrating that there were no HSCs in mutant fetal liver that could account for the observed results. These results also indicate that *Cbfβ* plays an important role in B-1 and MZ B cell development and expansion as reported in B-2 cells[36].

Taken all together, we suggest that YS/P-Sp hemogenic ECs produce B-1 progenitors, which seed the fetal liver to develop into the B-1 cell lineage in the fetal liver and contribute to the postnatal B-1 cell pool (Fig.1). In order to elucidate how much of the B-1 cell pool are YS/P-Sp derived, lineage-tracing models would be required to discriminate the YS/P-Sp- and fetal liver HSC-derived B-1 cells during postnatal development.

What kinds of lymphoid progenitors are produced at pre-HSC stage of mouse embryos? - Lineage-restricted progenitors vs. multi-potent progenitors?

Although B and T lymphoid potentials are detectable in YS and P-Sp at as early as E8.25 (4-6 sp stage)[29, 37], whether these lymphoid cells are derived from multipotent progenitors or lineage-restricted progenitors remains unknown. Before 26 sp, these B and T lymphoid cells are derived from CD41^{-}CD45^{-}VEcad^{+} hemogenic ECs, not from CD41^{+} or CD45^{+} hematopoietic cells[34]; thus this question is whether B and T lymphoid cells are derived from hemogenic ECs via multi-potent progenitors or lineage restricted progenitors (Fig. 2). A multipotent progenitor cell (MPP) that has a capacity of differentiation into erythro, myeloid, and lymphoid cells, but does not engraft in

recipient adult mice is first detectable in the E10 AGM region[38], fetal liver[39] and recently reported at late E9.5 YS[40]. In this sense, there are no MPPs reported before E9.5 stage, suggesting that these B and T lymphoid potentials are derived from lineage restricted progenitor cells before E9.5. Of note, these B and T lymphoid potentials are always observed with myeloid potential (although not by single cell assay). Thus, it is highly possible that B- or T- lineage-restricted progenitor cells directly differentiated from hemogenic EC that also retain myeloid potential.

In this sense, the myeloid and lymphoid lineage commitment branch is ambiguous during fetal hematopoiesis. A myeloid-based model has been previously suggested in 2001 by Drs. Katsura and Kawamoto[41], where three types of progenitors retain myeloid potential in myelo-lymphoid branches: myeloid-T-B-progenitors (p-MTB), myeloid-T progenitors (p-MT), and myeloid-B progenitors (p-MB). This model is based on the results of multi-lineage progenitor assays that allow clonal analysis of developmental potential of fetal liver progenitor cells using a modified fetal thymic organ culture (FTOC) system. When single E11.5 c-kit$^+$CD45$^+$ fetal liver cells are plated in the MLP assay culture, 6 types of progenitor cells were detected such as p-Multi (MTB), p-MT, p-MB, p-M, p-T, and p-B, demonstrating that lymphoid progenitors retain myeloid potential[38, 39]. Thus, this model is compatible with the B-1 progenitor cells that retain B-1 and myeloid potential as mentioned in the previous section. Similarly, Dr. Jacobsen's group has reported the presence of Rag1$^+$ Immune restricted progenitor cells that possess myelo-lymphoid potential (but not erythro-megakaryocyte potential) in the fetal liver[42] and seem to be equivalent cell types to those reported by Drs. Katsura and Kawamoto's group[39]. They have also shown Rag1cre$^+$YFP$^+$Mac1$^+$ myeloid cells in the fetal liver: this population declines with advancing age, and is greatly diminished in the adult BM[42]. This result strongly implies that Rag1cre$^+$YFP$^+$Mac1$^+$ myeloid cells may be derived from B-1 progenitor cells or bipotent myeloid-B progenitor cells in the fetal liver as previously reported[35, 43]. Interestingly a recent report has also demonstrated the presence of Rag1cre$^+$YFP$^+$Mac1$^+$ myeloid cells in adult thymus[44], supporting a myeloid-based model in the adult immune system as well. Thus, the relationship between myeloid and lymphoid

lineages is not straightforward as was previously thought[45] especially in fetal hematopoiesis, and it would be interesting to understand how hemogenic ECs gain lymphoid or mulipotent potentials to produce immune lineage cells (Fig. 2).

Summary

We have reviewed the evidence for the layered immune system hypothesis of B cell development that includes the first wave of B-1 cell development derived from YS and P-Sp hemogenic ECs independent of any HSC contributions. Although the second wave of B cell production (dominant B-1 cell production) is considered to be fetal liver HSC-derived, recent data suggests that isolated and purified single fetal liver long-term HSC transplantation may not reconstitute the B-1a cell pool (presented by Dr. Ghosn at Merinoff World Congress 2014: B-1 cell Development and Function). Other questions also remain to be answered: What molecules are involved in B-1 cell lineage commitment? What is the precursor of the B-1 progenitor cells in the fetal liver stage other than hemogenic EC that emerge from the YS and P-Sp? How and where do fetal liver B-1 progenitor cells mature into B-1a cells in vivo? Why does B-1 lineage potential in BM-derived HSC/CLP decline with advancing age? What types of cells are the counterpart of mouse B-1 cells in human subjects? It is apparent that we have just begun to unravel the complex pathways for the developmental origins and life-long homeostatic maintenance of the innate immune B-1 cells in mice. We anticipate the information learned will enhance our fundamental understanding of the important immune defense roles played by B-1 cells and perhaps enlighten our understanding of human immune defenses, particularly following bone marrow transplantation[46].

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References

1. Moore, M. A. & D. Metcalf. 1970. Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. Br J Haematol. 18: 279-296.

2. Lin, Y., M. C. Yoder & M. Yoshimoto. 2014. Lymphoid progenitor emergence in the murine embryo and yolk sac precedes stem cell detection. Stem Cells Dev. 23: 1168-1177.

3. Lux, C. T.*, et al.* 2008. All primitive and definitive hematopoietic progenitor cells emerging before E10 in the mouse embryo are products of the yolk sac. Blood. 111: 3435-3438.

4. Muller, A. M.*, et al.* 1994. Development of hematopoietic stem cell activity in the mouse embryo. Immunity. 1: 291-301.

5. Medvinsky, A. & E. Dzierzak. 1996. Definitive hematopoiesis is autonomously initiated by the AGM region. Cell. 86: 897-906.

6. North, T.*, et al.* 1999. Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. Development. 126: 2563-2575.

7. de Bruijn, M. F.*, et al.* 2002. Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. Immunity. 16: 673-683.

8. North, T. E.*, et al.* 2002. Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. Immunity. 16: 661-672.

9. Boisset, J. C.*, et al.* 2010. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature. 464: 116-120.

10. Jaffredo, T.*, et al.* 1998. Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. Development. 125: 4575-4583.

11. Nishikawa, S. I.*, et al.* 1998. In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. Immunity. 8: 761-769.

12. Chen, M. J.*, et al.* 2009. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. Nature. 457: 887-891.

13. Lancrin, C.*, et al.* 2009. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. Nature. 457: 892-895.

14. Eilken, H. M., S. Nishikawa & T. Schroeder. 2009. Continuous single-cell imaging of blood generation from haemogenic endothelium. Nature. 457: 896-900.

15. Zovein, A. C.*, et al.* 2008. Fate tracing reveals the endothelial origin of hematopoietic stem cells. Cell Stem Cell. 3: 625-636.

16. Tober, J.*, et al.* 2013. Distinct temporal requirements for Runx1 in hematopoietic progenitors and stem cells. Development. 140: 3765-3776.

17. Okuda, T.*, et al.* 1996. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell. 84: 321-330.

18. Schulz, C.*, et al.* 2012. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science. 336: 86-90.

19. Herzenberg, L. A. 1989. Toward a layered immune system. Cell. 59: 953-954.

20. Hardy, R. R. & K. Hayakawa. 2001. B cell development pathways. Annu Rev Immunol. 19: 595-621.

21. Hardy, R. R. & K. Hayakawa. 1991. A developmental switch in B lymphopoiesis. Proc Natl Acad Sci U S A. 88: 11550-11554.

22. Carvalho, T. L.*, et al.* 2001. Arrested B lymphopoiesis and persistence of activated B cells in adult interleukin 7(-/-)- mice. J Exp Med. 194: 1141-1150.

23. Hao, Z. & K. Rajewsky. 2001. Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. J Exp Med. 194: 1151-1164.

24. Montecino-Rodriguez, E., H. Leathers & K. Dorshkind. 2006. Identification of a B-1 B cell-specified progenitor. Nat Immunol. 7: 293-301.

25. Ghosn, E. E.*, et al.* 2012. Distinct B-cell lineage commitment distinguishes adult bone marrow hematopoietic stem cells. Proc Natl Acad Sci U S A. 109: 5394-5398.

26. de Andres, B.*, et al.* 2002. The first 3 days of B-cell development in the mouse embryo. Blood. 100: 4074-4081.

27. Cumano, A., F. Dieterlen-Lievre & I. Godin. 1996. Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. Cell. 86: 907-916.

28. Yokota, T.*, et al.* 2006. Tracing the first waves of lymphopoiesis in mice. Development. 133: 2041-2051.

29. Yoshimoto, M.*, et al.* 2011. Embryonic day 9 yolk sac and intra-embryonic hemogenic endothelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. Proceedings of the National Academy of Sciences of the United States of America. 108: 1468-1473.

30. Montecino-Rodriguez, E. & K. Dorshkind. 2012. B-1 B cell development in the fetus and adult. Immunity. 36: 13-21.

31. Miller, J.*, et al.* 2002. The core-binding factor beta subunit is required for bone formation and hematopoietic maturation. Nat Genet. 32: 645-649.

32. Chen, M. J.*, et al.* 2011. Erythroid/myeloid progenitors and hematopoietic stem cells originate from distinct populations of endothelial cells. Cell stem cell. 9: 541-552.

33. Wang, Q.*, et al.* 1996. The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo. Cell. 87: 697-708.

34. Kobayashi, M.*, et al.* 2014. Functional B-1 progenitor cells are present in the hematopoietic stem cell-deficient embryo and depend on Cbfbeta for their development. Proc Natl Acad Sci U S A. 111: 12151-12156.

35. Montecino-Rodriguez, E., H. Leathers & K. Dorshkind. 2001. Bipotential B-macrophage progenitors are present in adult bone marrow. Nat Immunol. 2: 83-88.

36. Seo, W.*, et al.* 2012. Runx1-Cbfbeta facilitates early B lymphocyte development by regulating expression of Ebf1. J Exp Med. 209: 1255-1262.

37. Yoshimoto, M.*, et al.* 2012. Autonomous murine T-cell progenitor production in the extra-embryonic yolk sac before HSC emergence. Blood. 119: 5706-5714.

38. Ohmura, K.*, et al.* 1999. Emergence of T, B, and myeloid lineage-committed as well as multipotent hemopoietic progenitors in the aorta-gonad-mesonephros region of day 10 fetuses of the mouse. J Immunol. 163: 4788-4795.

39. Kawamoto, H.*, et al.* 2000. T cell progenitors emerge earlier than B cell progenitors in the murine fetal liver. Immunity. 12: 441-450.

40. Inlay, M. A.*, et al.* 2014. Identification of Multipotent Progenitors that Emerge Prior to Hematopoietic Stem Cells in Embryonic Development. Stem cell reports. 2: 457-472.

41. Kawamoto, H.*, et al.* 2011. A map for lineage restriction of progenitors during hematopoiesis: the essence of the myeloid-based model. Immunol Rev. 238: 23-36.

42. Boiers, C.*, et al.* 2013. Lymphomyeloid contribution of an immune-restricted progenitor emerging prior to definitive hematopoietic stem cells. Cell Stem Cell. 13: 535-548.

43. Cumano, A.*, et al.* 1992. Bipotential precursors of B cells and macrophages in murine fetal liver. Nature. 356: 612-615.

44. De Obaldia, M. E., J. J. Bell & A. Bhandoola. 2013. Early T-cell progenitors are the major granulocyte precursors in the adult mouse thymus. Blood. 121: 64-71.

45. Kondo, M., I. L. Weissman & K. Akashi. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell. 91: 661-672.

46. Moins-Teisserenc, H.*, et al.* 2013. CD19(+)CD5(+) B Cells and B1-Like Cells Following Allogeneic Hematopoietic Stem Cell Transplantation. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 19: 988-991.

47. Rybtsov, S.*, et al.* 2011. Hierarchical organization and early hematopoietic specification of the developing HSC lineage in the AGM region. J Exp Med. 208: 1305-1315.

Figure legends

Figure 1. The first and second waves of B-1 cell development in the mouse embryo.

Hemogenic ECs in the YS/P-Sp produce B-1 progenitor cells prior to HSC emergence. These progenitor cells seed fetal liver (the first wave). Around at E11, the first HSCs emerge from hemogenic ECs at AGM region and also seed fetal liver and expand. Fetal liver B-1 progenitor cells derived from YS hemogenic EC (the first wave) and HSC (the second wave) mature into the peritoneal B-1 cells after birth.

Figure 2. A hypothesis of hemogenic EC maturation and their production of various progenitor cells.

In early stage of YS (E8-9.5), hemogenic ECs produce lineage restricted progenitors such as EMP, B/B-myeloid, and T/T-myeloid progenitor cells. Before E10, hemogenic ECs produce MPPs that can produce erythro-myeloid, lymphoid cells and these MPPs may mature into HSCs in vivo[47]. Also, hemogenic ECs at AGM region may further mature into HSC-producing ECs.

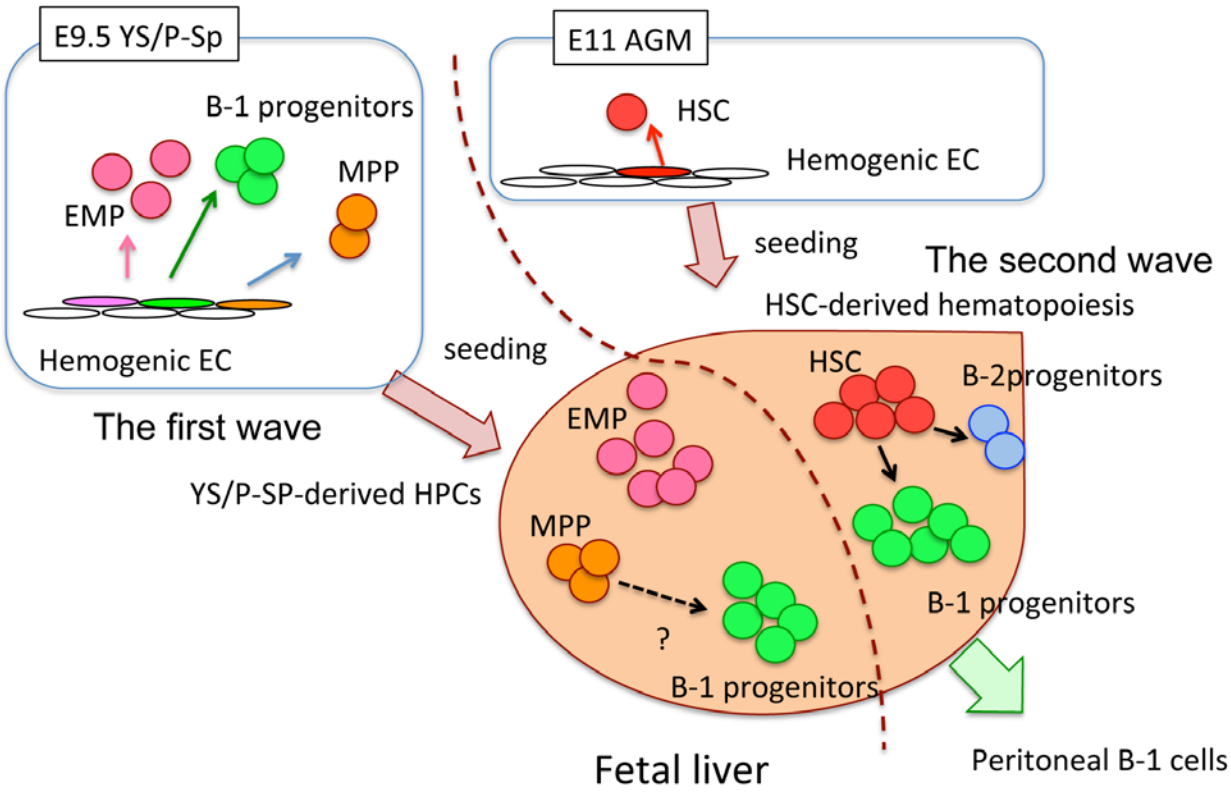
E9.5 YS/P-Sp
B-1 progenitors
MPP
EMP
Hemogenic EC
The first wave
E11 AGM
HSC
Hemogenic EC
seeding
The second wave
HSC-derived hematopoiesis
seeding
HSC
B-2progenitors
EMP
YS/P-SP-derived HPCs
MPP
?
B-1 progenitors
B-1 progenitors
Fetal liver
Peritoneal B-1 cells

Figure 1

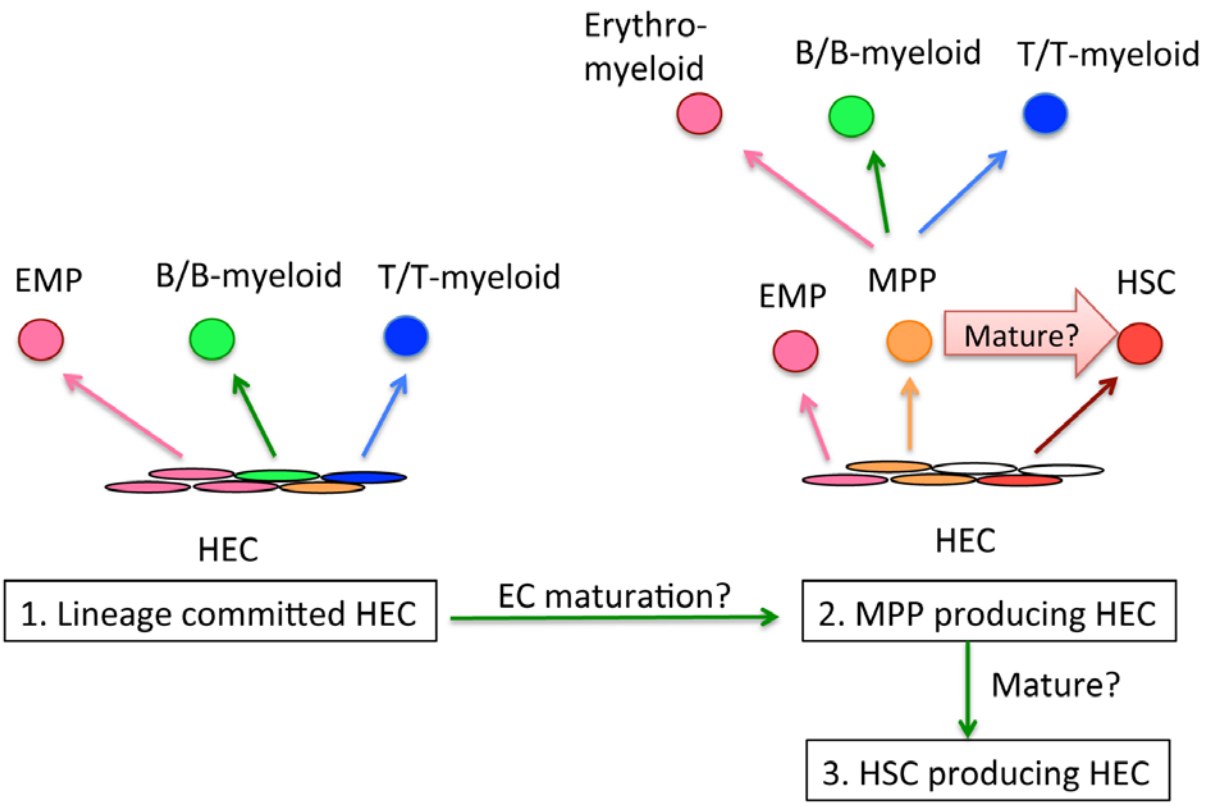
Erythro-
myeloid
B/B-myeloid
T/T-myeloid
EMP
B/B-myeloid
T/T-myeloid
EMP
MPP
HSC
Mature?
HEC
HEC
1. Lineage committed HEC
EC maturation?
2. MPP producing HEC
Mature?
3. HSC producing HEC

Figure 2